



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, 1/02	A1	(11) International Publication Number: WO 99/53092 (43) International Publication Date: 21 October 1999 (21.10.99)
<p>(21) International Application Number: PCT/BE99/00049</p> <p>(22) International Filing Date: 12 April 1999 (12.04.99)</p> <p>(30) Priority Data: 98870081.1 14 April 1998 (14.04.98) EP</p> <p>(71) Applicant (for all designated States except US): VLAAMSE INSTELLING VOOR TECHNOLOGISCH ONDERZOEK (VITO) [BE/BE]; Boeretang 200, B-2400 Mol (BE).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): VAN DER LELIE, Daniël [NL/BE]; Boeretang 246, B-2400 Mol (BE). REGNIERS, Luc, Agnes, Louis, Jean, Bosco [BE/BE]; Mezestraat 8, B-2480 Dessel (BE). TAGHAVI, Safiyh [BE/BE]; Quai de Rome 85, Boîte postale 71, B-4000 Liège (BE). CORBISIER, Philippe, Gilbert, Ghislain [BE/BE]; Boeretang 222, B-2400 Mol (BE). VERSCHAEVE, Luc, Philippine, Edouard [BE/BE]; Gebroeders Goemaerelaan 36, B-1160 Brussels (BE).</p> <p>(74) Agents: VAN MALDEREN, Eric et al.; Office van Malderen, Place Reine Fabiola 6/1, B-1083 Brussels (BE).</p>		<p>(81) Designated States: AU, BR, CA, CN, JP, MX, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published With international search report. With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.</p>
<p>(54) Title: DIAGNOSTIC SYSTEM AND METHOD FOR DETERMINING THE PRESENCE OF A GENOTOXIC COMPOUND IN A SAMPLE</p>		
<p>(57) Abstract</p> <p>This invention concerns a diagnostic system made of: a transformed microorganism capable of an increased reporter activity upon exposure to an environmental insult, said microorganism having a stress inducible promoter sequence being operatively linked to a reporter encoding nucleic acid sequence encoding a reporter molecule resulting in a signal that can be assayed, and of a transformed microorganism having a constitutive and non stress inducible promoter sequence being operatively linked to a reporter encoding nucleic acid sequence encoding a reporter molecule resulting in a signal that can be assayed.</p> <div data-bbox="768 1192 1377 1955"> <pre> graph TD SN[Signal/Noise] --> Pr1[Pr1-sensor] SN --> RecN[RecN(2-4)-sensor] Pr1 -- "< 0.8" --> T1[TOXIC] Pr1 -- "0.8-1.2" --> NE1[No effect] Pr1 -- "> 1.2" --> I1[Induction] RecN -- "< 0.8" --> T2[TOXIC] RecN -- "0.8-1.2" --> NE2[No effect] RecN -- "> 1.2" --> I2[induction] T1 --> R[Ratio] NE1 --> R I1 --> R T2 --> R NE2 --> R I2 --> R R -- "< 1.5" --> NS[Not significant] R -- "> 1.5" --> G[Genotoxic] </pre> </div>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

5

DIAGNOSTIC SYSTEM AND METHOD FOR DETERMINING THE PRESENCE
OF A GENOTOXIC COMPOUND IN A SAMPLE

10 Field of the invention

The present invention is related to a diagnostic system and to a method for determining the presence of a genotoxic compound in a sample.

15 Background of the invention and state of the art

The International Patent Application PCT/EP96/01745 describes a recombinant nucleic acid sequence, a host micro-organism comprising said nucleic acid sequence and the use of said host micro-organism for
20 detecting the presence of genotoxic compounds in a sample. Said bacterial genotoxicity test is based on bioluminescence and allows an easy, very rapid and low cost detection of genotoxic compounds. The test was shown to be at least as sensitive as the Ames test and SOS-chromotest
25 and to allow genotoxicity kinetics measurements as well as a simultaneous evaluation of the toxicity of the test compound or -material (van der Lelie et al., 1997). This new test, referred to as the VITOTOX[®] test was therefore considered to be a valuable short-term genotoxicity and
30 toxicity test for many different purposes.

The test is based on bacteria that contain the *lux* operon of *Vibrio fischeri* under transcriptional control of the *recN* gene, that is part of the SOS-system. After incubation of the bacteria in the presence of a

genotoxic compound, the *recN* promoter is derepressed, resulting in expression of the *lux* operon. This expression results in light production in function of genotoxicity. Originally, the test was performed with different modified
5 *Escherichia coli* and *Salmonella typhimurium* strains. *Salmonella typhimurium* strains (TA98, TA100 and TA104) were further used, as the bacteria are well known for mutagenicity testing and because the same bacteria could also be used for a classical Ames test, should this be
10 required. The construct using a *recN* promoter up mutation (*recN* 2-4) gave the best results in all strains. Furthermore, as all *Salmonella* strains gave very comparable results, it has been proposed to further use only the TA104 constructs [called TA104 (*recN*2-4)] as it was shown to be
15 sometimes a little more sensitive than the other hybrid strains (van der Lelie et al., 1997).

However, some compounds act directly on the light production (e.g. aldehydes, organic solvents) or enhance the metabolism of the bacteria creating false-
20 positive results.

Aims of the invention

The present invention aims to provide a new diagnostic system and method for the detection of
25 environmental insults such as the presence of a genotoxic compound into a sample, which do not present the drawbacks of the state of the art.

A main aim of the present invention is to provide a new diagnostic system and method which will
30 exclude false-positive and false-negative results.

A further aim of the present invention is to provide such a diagnostic system and method which could be used for the screening of genotoxic compounds obtained in the chemical, cosmetical or pharmaceutical industry field,

as a prevalidation study of intermediate or active chemical, cosmetical and/or pharmaceutical compounds.

A last aim of the present invention is to provide such a diagnostic system and method which allow an automatic screening upon very small volume of sample.

Description of the invention

The present invention is related to a diagnostic system made of

- 10 • a transformed micro-organism capable of an increased reporter activity upon exposure to an environmental insult, preferably exposure to a genotoxic compound, said micro-organism having a stress inducible promoter sequence, preferably a promoter sequence which is
15 inducible by a genotoxic compound, said promoter sequence being operatively linked to a reporter encoding nucleic acid sequence encoding a reporter molecule resulting in a signal that can be assayed, and of
- a transformed micro-organism having a constitutive and
20 non-stress inducible promoter sequence, preferably a constitutive promoter sequence which is not inducible by said genotoxic compound, said promoter sequence being operatively linked to a reporter encoding nucleic acid sequence encoding a reporter molecule resulting in a
25 signal that can be assayed.

Preferably, both transformed micro-organisms are bioluminescent micro-organisms and the signal can be assayed as light production. Other possibilities are the peroxydase, alkaline phosphatase, β -gal and gus genetic
30 sequence, where the signal will be assayed as a colorimetric modification or a chemiluminescent light production by using a colorimetry analyser or a photomultiplier device.

Advantageously, the diagnostic system according to the invention is made of two transformed bioluminescent micro-organisms, the first bioluminescent micro-organism is "activated" in the presence of a
5 genotoxic compound and results in a signal that can be assayed as light production, while the light production of the second bioluminescent micro-organism is not influenced by the presence of a genotoxic compound in the sample.

Nucleic acid sequence encoding a reporter
10 resulting in a signal that can be assayed as light production has been already described in the state of the art. Preferably, the transformed bioluminescent micro-organisms according to the invention comprise luciferase A and B genes, also identified as expressive lux genes
15 complex, comprising the luxA and luxB genes or a translational luxAB fusion gene. The transformed bioluminescent micro-organisms may also comprise the luciferase C, D and E genes, required for production of limiting fatty acid substrate that is used in recycling.

20 According to a preferred embodiment of the present invention, the diagnostic system comprises a transformed bioluminescent micro-organism having a constitutive and non stress inducible promoter sequence with a Sigma 70 consensus sequence (TTGACA(-35).....17/18 bp
25TATAAT(-10)) and whose transcription is not regulated positively or negatively at the promoter level. Said promoter consensus is described in Hoopes BC and McClure WR (1987): Strategies in Regulation of Transcriptional Initiation; in "Escherichia Coli and Salmonella
30 typhimurium, Cellular and Molecular Biology, FC Neidhart, JL Ingraham, KB Low, B Maganasik, M Schaechter, HE Umbaeger (eds.), American Society for Microbiology, Washington D.C., pp 1231-1240.

According to a preferred embodiment of the

present invention, the transformed bioluminescent micro-organisms are selected from the group consisting of *E. coli* or *Salmonella typhimurium*, and are advantageously suitable Ames test micro-organism(s), preferably selected from the group consisting of TA98, TA100, TA102, TA104, TA1535, TA1538, TA7001 to TA7006, and TA7041 to TA7046, and/or having the micro-organism deposit number LMG P-18318. The micro-organism with deposit number LMG P-18318 will be identified hereafter as the "pr1" strain.

Advantageously, the stress inducible promoter sequence in the transformed bioluminescent micro-organism of the diagnostic system according to the invention is selected from the group consisting of *groEL*, *dnaK*, *grpE*, *phoA*, *glnA*, *lon*, *lysU*, *rpoD*, *clpB*, *clpP*, *uspA*, *katG*, *uvrA*, *frdA*, *micF*, *fabA*, *lac*, *his*, *sodA*, *sodB*, *soi-28*, *recA*, *xthA*, *narG*, *recF*, *recJ*, *recN*, *recO*, *recQ*, *ruv*, *uvrD*, *ars*, *cad*, *mer*, *pco*, and *sfiA*.

According to a preferred embodiment of the present invention, the stress inducible promoter sequence is *recN*, advantageously *recN2-4*. Said micro-organism will be identified hereafter as the "recN2-4" strain

In a preferred embodiment, the diagnostic system according to the invention comprises a transformed bioluminescent micro-organism having a stress inducible promoter sequence being a SOS regulator promoter sequence, having preferably an induction ration higher than 40, and comprising advantageously a mutation improving the promoter strength or regulation, said mutation not destroying the SOS regulation.

A specific example of mutated *recN* promoter sequence is described in the International Patent Application PCT/EP96/01745, incorporated hereafter by reference.

Said stress inducible promoter sequence

comprises also a promoter up-mutation, preferably a promoter up-mutation in the -35 region of said promoter, described in the International Patent Application PCT/EP96/01745, incorporated hereafter by reference.

5 The present invention is also related to a diagnostic kit comprising the elements of the diagnostic system according to the invention and possibly the necessary additional reactants, diluants and/or solid supports for said diagnostic such as a buffer solution, a
10 solution comprising a specific marker such as the X-gal (5-bromo-4-chloro-3-indoyl- β -galactoside) for a diagnostic based upon the use of a β -gal genetic sequence, the luminol for a diagnostic based upon the use of a peroxydase genetic sequence, etc...

15 The present invention is also related to a general method for the diagnostic of an environmental stress or insult, preferably for determining the presence of a genotoxic compound in a sample. Said method comprises the steps of exposing the diagnostic system according to
20 the invention to said environmental insult (preferably comprising the steps of exposing the diagnostic system to the genotoxic compound present in the sample) and measuring a signal, preferably a change in luminescence of said diagnostic system.

25 The present invention is also related to a method for determining the kinetics of genotoxicity of a compound into a sample, based upon the above-identified method, wherein the measuring of luminescence of both inducible and constitutive transformed micro-organisms
30 occurs at multiple points in time, preferably continuously, and in addition carrying out the step of determining the signal-to-noise (S/N) ratio for the transformed micro-organisms at said point and time, dividing the S/N ratio of

the inducible micro-organism by that of the constitutive micro-organisms and plotting these data, said plotting representing the corrected kinetics of genotoxicity of the genotoxic compound in the sample.

5 Advantageously, the diagnostic system and method according to the invention can be combined with the Ames and/or SOS chromotest(s) and method(s), as described hereafter.

 The present invention is also related to an
10 analysis method of an environmental insult, comprising the steps of:

- performing the diagnostic of said environmental insult as above described,
- calculating the signal to noise ratio of both the
15 transformed micro-organisms,
- classifying said environmental insult as toxic if at least one of the calculated signal to noise ratios is lower than 0.8,
- classifying said environmental insult as having no
20 effect if the signal to noise ratio of the micro-organism comprising the stress-inducible promoter sequence lies between 0.8 and 1.2, and
- classifying said environmental insult as inducing and
25 genotoxic if the signal to noise ratio of the micro-organism comprising the stress-inducible promoter sequence is higher than 1.2 and is at least 50 % higher than the signal to noise ratio of the micro-organism comprising the constitutive promoter sequence.

 The present invention is also related to an
30 installation for determining if a compound in a sample is genotoxic and/or toxic, comprising a diagnostic system as above described induced by said sample, a detection system comprising a detection apparatus able to assay the signal from said diagnostic system, said detection system being

connected to a computer programmed to carry out the analysis method as above described.

The present invention will be described in
5 detailed in the non-limiting following examples, in reference to the following figures.

Short description of the drawings

- Figure 1 represents signal-to-noise ratio for
10 epichlorohydrine in the *recN2-4* and *pr1* strains.
- Figure 2 represents signal-to-noise ratio for ZnCl_2 in the *recN2-4* and *pr1* strains.
- Figure 3 represents signal-to-noise ratio for sodium
15 azide in the *recN2-4* and *pr1* strains.
- Figure 4 represents signal-to-noise ratio for nifuroxazide in the *recN2-4* and *pr1* strains.
- Figure 5 represents a determination chart to
20 differentiate the possible outcomes of a diagnostic test according to the invention.
- Figure 6 represents an installation according to the invention.

Detailed description of the invention

25 MATERIALS AND METHODS

Ames test and SOS chromotest

The SOS chromotest and the Ames test are well known and widely used bacterial genotoxicity tests (e.g., Quillardet & Hofnung, 1993; Mersch-Sundermann et al., 1994;
30 Mortelmans et al., 1986). The "classical" Ames test was routinely performed with *Salmonella typhimurium* strains TA98 and TA100 using the standard protocol described by Maron and Ames (1983). The SOS-chromotest was purchased as

a test kit from Organics ltd (Yavne, Israel). The test was performed as indicated in the manufacturers instructions.

The VITOTOX[®] test

5 Salmonella typhimurium strains

The *recN* promotor region, that was part of the *E. coli recN* gene (Rostas et al., 1987) contains two LexA binding sites. One LexA binding site overlaps with the -35 region while the second overlaps with the -10 region and the transcription start point of the *recN* promoter. The *E. coli recN* promoter was cloned upstream of the *luxCDABE* operon of the expression vector pMOL877 (van der Lelie et al., 1997). This resulted in pMOL1066. Since the *recN* promoter is under control of the bacterial SOS system, the expression of the *lux* operon became SOS regulated. This results in light production in the presence of genotoxins. Some *recN* promoter derivatives were also cloned in pMOL877. One lacking the LexA2 site resulted in pMOL1067, one having a promoter up mutation (pMOL1068) and one being a combination of both (pMOL1069). All constructs were introduced in the Ames test strains TA98, TA100 and TA104 and were able to detect genotoxic compounds. However, as the best results were obtained with strain TA104 (pMOL1068) this strain was further used in the so-called VITOTOX[®] test. It was extensively described before and was designated as TA104 *recN*2-4 as it contains the *recN*2-4 PCR fragment (van der Lelie et al., 1997). Besides TA104 *recN*2-4 (the tester strain) the so-called TA104 *prl* strain is now added as a "control strain". Plasmid pMOL1046 was constructed by cloning at random *EcoRI* digested DNA fragments from *Alcaligenes eutrophus* CH34 in the *luxCDABE*-expression vector (pMOL877). *A. eutrophus* CH34 (ATCC 43123) is a gram-negative non-pathogenic soil bacterium derived

from a decantation tank of a zinc factory (Mergeay et al., 1985, J.Bact). After transformation into *E. coli* 1106 (Murray et al., 1977, Mol. Gen.Genet.), clones were selected for light production. The best constitutively
5 light emitting clone, giving the highest light production as quantified in a luminometer, was then selected out of the different plasmid transformants (=plasmid pMOL1046) and introduced into the *S. typhimurium* strain TA104. This was named the "pr1" strain. It contains *luxCDABE*-genes under
10 control of a constitutive promoter so that the light production is not influenced by genotoxic compounds. The pr1 strain is used in parallel with the recN2-4 strain and cultivated and treated in exactly the same way.

15 Test procedure

1. Cultures

Bacteria were incubated overnight on a rotative shaker at 37°C in 869 medium; this is a normal growth medium equivalent to Luria Broth medium supplemented
20 with extra CaCl₂ to allow optimal bacterial growth and is described in van der Lelie et al.(1997, Mutation Res.). The next morning the bacterial suspension was diluted 10 times in 869 medium and 50µl of the dilution was then inoculated in 2.5ml of 869 medium and incubated for one more hour at
25 37°C on a rotative shaker (170 rpm).

2. Preparation of the 96-well plates

In the meantime 96-well plates were prepared so as to contain 10µl of either the solvent, different
30 concentrations of the test compound or the positive control for genotoxicity testing with (2-AF) or without (4-NQO) S9-mix. The S9 mixture, prepared according to Maron and Ames (1983, Mutation Res.), is used to detect the presence of compounds that only become genotoxic after metabolic

activation. The S9-mix was prepared freshly before use. For tests with S9-mix, 140 μ l of the bacteria (*recN2-4* or *pr1*) was added to 860 μ l poor 869 medium and 400 μ l S9-mix. From this mixture 90 μ l was then added to the 10 μ l solution already present in the wells. For tests without S9-mix, 1260 μ l of poor 869 medium was added to 140 μ l of the bacterial suspension and 90 μ l of the mixture was then transferred to wells containing 10 μ l of the test compound or controls.

10

3. Genotoxicity and toxicity measurements

A 96-well microplate luminometer (Luminoscan from Berthold) was used for the measurements of the light production following exposure to the test compounds. Light emission from each of the wells was measured every 5 minutes during 5 hours (30°C, 1sec/well, 60 cycles of 300sec each). After completing the measurements, the data were transferred into an MS Excel macro sheet and the signal-to-noise ratio (S/N), being the light production of exposed cells divided by the light production of non-exposed cells, was calculated for each measurement. A compound was considered genotoxic when the S/N was higher than 2 for at least two concentrations and when a clear dose-effect relationship was observed. In the second experiment in which the TA104 *pr1* strain was introduced, the S/N was calculated for the *RecN2-4* and *pr1* strains separately as well as the ratio between the maximum and mean S/N values of the *recN2-4* and *pr1* strains (*rec/pr1*). All calculations were performed between 60 and 240 minutes of incubation. Here, a compound is considered genotoxic when the S/N in the *recN2-4* strain is greater than 1.5x the solvent control value (and not "2" as was initially done), but only when this increase is not accompanied by a comparable increase in the *pr1* strain (that would indicate

a non-genotoxic induction mechanism). A compound is genotoxic when the S/N ratio's of the *recN2-4* over the *prl* strain is equal or higher than 1.5 (limit set on experimental grounds). In this way "false positive" results
5 can be avoided.

"False negative" results can be identified when the S/N ratio of the *recN2-4* strain remains between 0.8 and 1.2, whereas the S/N ratio of the *prl* sensor decreases below 0.8. This is typically the case for samples
10 being genotoxic as well as toxic at the same time.

The *prl* strain is furthermore also very valuable for toxicity evaluation. Bacterial toxicity is assumed when the light emission is substantially decreasing in a dose-dependent way and attains S/N values lower than
15 0.8 within the first 30 minutes of the test.

Automatisation of the system is possible, using an installation as described in fig. 6. An detection apparatus (2) comprising at least a detector for the signal
20 that is emitted from a diagnostic system as above described, is linked to a computer (1) which receives and processes the detector data according to the analysis method as above described and as clarified in figure 5. This way, an automatised analysis of samples and
25 classification as toxic and/or genotoxic is possible.

Test compounds

A number of commercially available well known compounds that were evaluated before with the TA104 *recN2-4*
30 strain alone (see van der Lelie et al., 1997) were re-evaluated in the present work using the TA104 *recN2-4* and TA104 *prl* strain. They are given in table 1.

concentration at which

S/R's of rec/pri >1.5

COMPOUND*	S9**	DOSE RANGE TESTED	S/R>2 (recN2-4)	MAX	MEAN	Toxic (pri)
Furazolidone	-	0.125-32ppb	0.25ppb	0.5ppb	0.25ppb	-
4NQO	-	0.4-102ppb	0.8ppb	1.6ppb	0.8ppb	-
Nifuroxazide	-	2-256ppb	8ppb	16ppb	8ppb	+
MMC	-	3.9-500ppb	31.2ppb	31.2ppb	15.6ppb	-
3-Nitrofluoranthene	-	7.9-1000ppb	15.7ppb	31.3ppb	15.7ppb	-
3-Nitrofluoranthene	+25	7.9-1000ppb	15.7ppb	31.2ppb	15.7ppb	-
Nifuroxazide	-	0.04-5.12ppm	0.04ppm	0.04ppm	0.04ppm	+
3Nitrofluoranthene	-	0.004-0.512ppm	0.032ppm	0.064ppm	0.032ppm	-
Carbadox	-	0.04-5.12ppm	0.08ppm	0.08ppm	0.04ppm	-
Nalidixic acid	-	0.02-2.56ppm	0.16ppm	0.16ppm	0.16ppm	+
2,4,5,7Tetranitro-9-fluorenone	-	0.01-1.28ppm	0.08ppm	0.16ppm	0.04ppm	+

B(a)P	+25	0.025-6.4ppm	0.2ppm	0.2ppm	0.1pm	-
2AF	+25	0.2-3.2ppm	0.2ppm	0.2ppm	0.2ppm	-
B(a)P	+25	0.1-1.6ppm	0.2ppm	0.2pm	0.2pm	-
2,7Dinitrofluoreen	+25	0.04-10ppm	0.62ppm	0.62ppm	0.62ppm	-
B(a)P	+100	0.1-12.8ppm	0.8ppm	0.8ppm	0.4ppm	-
ICR 191 Acridine	-	0.02-2.5ppm	0.62ppm	1.25ppm	0.31ppm	-
a-Naphtylamine	+25	0.08-10ppm	5ppm	2.5ppm	2.5ppm	-
4Nitro-O-	-	0.79-100ppm	3.1ppm	3.1ppm	1.57ppm	+
Phenylenediamine						
Fluoranthene	+100	3.1-400ppm	3.1ppm	3.1ppm	3.1ppm	-
H2O2	-	0.25-32ppm	2ppm	4ppm	2ppm	+
K2Cr2O7	-	0.5-64ppm	4ppm	4ppm	4ppm	+
Phenanthrene	+100	3.1-400ppm	6.2ppm	6.2ppm	6.2ppm	+
MMS	-	4-64ppm	16ppm	8ppm	8ppm	-
MMS	-	0.5-128ppm	8ppm	8ppm	8ppm	-

Chrysene	+100	0.15-20ppm	-	10ppm	5ppm	-
4Nitro-o-	+25	0.79-100ppm	12.5ppm	12.5ppm	12.5ppm	+
Phenylenediamine						
N-Nitrosodiethylamine	+25	3.25-480ppm	120ppm	120ppm	240ppm	-
Epichlorohydrine	-	4-512ppm	120ppm	256ppm	120ppm	-
EMS	-	8-1024ppm	256ppm	256ppm	256ppm	-
Epichlorohydrine	-	8-1024ppm	256ppm	512ppm	128ppm	+
ZnCl2	-	0.5-64ppm	-	-	-	+
CdCl2	-	0.78-100ppm	-	-	-	+
Coumermycine A1	-	1.56-200ppm	-	-	-	+
Sodium azide (NaN ₃)	-	2-256ppm	-	-	-	+
2,7Dinitrofluoreen	-	0.04-10ppm	-	-	-	-
a-Naphtylamine	-	0.08-10ppm	-	-	-	-

Table 1: VITOTOX test results for selected chemicals (* some chemicals were investigated several times in different dose-ranges or conditions; ** μ l/ml of S9-mix used at incubation)

RESULTS

Earlier reported results with the VITOTOX[®]-test were obtained in *Salmonella typhimurium* strain TA104
5 RecN2-4 (van der Lelie et al., 1997). In order to further improve the test we introduced the *prl* strain. Results obtained in the RecN2-4 strain should be evaluated in comparison with the results obtained in the *prl* strain where an increased light production cannot be due to a
10 genotoxic event. An increased light production in the recN2-4 strain can therefore only be interpreted as an indication of genotoxicity when this is not accompanied by a comparable increase in light production in the *prl* strain.

15 Using both bacterial strains we re-evaluated a number of the earlier studied chemicals. The results are given in table 1. The table gives the concentrations at which the maximal S/N value in the recN2-4 tester strain becomes ≥ 2 , as well as the concentrations where the ratio
20 between the maximal and mean recN2-4 S/N and *prl* S/N reaches 1.5 or more (*rec/prl*). Finally the table also indicates the presence of toxicity within the dose-range tested and as evaluated by the *prl* S/N curves. It can be seen that known genotoxic compounds were indeed evaluated
25 as genotoxic whereas non-genotoxic compounds did not show the required S/N ratio's in the given dose-ranges. A few examples of the results were graphically represented in figures 1-4 (examples of tests without S9mix). Figure 1 gives the S/N curves for epichlorohydrine in the recN2-4
30 and *prl* strains. In the recN2-4 strain the S/N values became greater than 2 at the dose of 256 ppm, whereas S/N values in the *prl* strain did not greatly deflect from 1. Figure 2 gives the results for ZnCl₂ that is non-genotoxic

but toxic for *Salmonella typhimurium* TA104 at concentrations higher than 7.4 μ M. Sodium azide is given as a third example in figure 3. Here the S/N ratio was considerably greater than 2 in both the *recN2-4* and *prl* strains and indications of toxicity are obtained with time (S/N<0.8). Figure 4 illustrates the results that were obtained for nifuroxazide. Referring to the *recN2-4* strain lower doses were apparently more genotoxic than higher doses but the *prl* strain showed a dose-dependent decrease in light production indicating toxicity.

DISCUSSION

The VITOTOX[®]-test is a sensitive and rapid method to detect genotoxic compounds (van der Lelie et al., 1997). However, if only the *recN2-4* strain is used (as was initially done) some misinterpretations were possible. The present invention is based upon the use of the *prl* strain. The added value of the *prl* strain was illustrated by a few examples. In figure 1 an example is given of a genotoxic compound (epichlorohydrine) that was not toxic in the given dose-range. Based on the results obtained from the *recN2-4* strain alone one may conclude that the compound was genotoxic as a dose-dependent increase in light production was observed that exceeded the "noise" value by more than a factor of two (S/N>2). Inclusion of the *prl* strain only confirmed this evaluation. If an increased light production was found in the *prl* strain we indeed should conclude that this was due to another induction mechanism than genotoxicity (e.g., increased cell proliferation which would enhance the "noise level" compared to that of unexposed cultures, etc.). But this was not the case. There was also no sign of toxicity as there was no decreased light production. This was clearly the case for ZnCl₂ as indicated by the curves of figure 2. The figure for the

recN2-4 strain shows that the dose of 3.7 μM is neither genotoxic nor toxic but at higher doses a decreased light emission is observed indicating a toxic effect followed by a certain recovery at the lower doses. This is confirmed by
5 the *prl* strain where recovery is even more visible at doses up to 58.8 μM ZnCl_2 . From 117.5 μM ZnCl_2 on, recovery is not possible anymore. ZnCl_2 was thus shown to be non genotoxic and only the lowest dose appeared to be devoid of any toxicity.

10 As indicated in the introduction and, materials and methods section the VITOTOX[®]-test is essentially based on detection of a SOS signal. It should therefore theoretically produce results that are more in agreement with the SOS chromotest than with the Ames test.
15 Yet, some differences were previously found. The Inventors have reported for example a "positive" response for sodium azide while this compound normally scores "negative" in the SOS chromotest (van der Lelie et al, 1997). One reason for this discrepancy could be that the VITOTOX[®]-test uses the
20 Ames-test's *Salmonella typhimurium* TA104 strain and therefore has the same characteristics, e.g., reduced DNA repair capability and increased permeability for complex molecules (as most pharmaceutical compounds). This is certainly a valid explanation in many instances but
25 probably not for a small molecule like sodium azide (N_3Na). Here another reason for the difference with SOS chromotest results could be anticipated. It could for example be that the increased light production as found in the *recN2-4* strain is due to another induction mechanism than
30 genotoxicity. With the introduction of the *prl* strain one is able to verify this assumption. As can be seen from figure 3 the observed light production in the *recN2-4* strain is not due to genotoxicity as increased light

emission is also observed in the *prl* strain and this can not be due to a SOS response. Sodium azide should therefore be interpreted as non-genotoxic in our test. It is a good example of a "compound" that would be a "false positive" when only the *recN2-4* strain was used.

The Inventors have tested and compared much more pharmaceutical compounds with the other bacterial tests. Results obtained by the different tests were most often completely in agreement with each other. The VITOTOX® test usually is also much more sensitive than the Ames or SOS chromotest as the minimal detectable concentrations are 1-100 times lower in the VITOTOX® test.

Another advantage of using the *prl* strain is that besides genotoxicity, toxicity can be better evaluated than with the *recN2-4* strain alone. A genotoxic compound that is investigated at dose levels around the toxicity threshold can show S/N ratio's in the *recN2-4* strain that are "intermediate" between genotoxicity and toxicity as increase in light production (due to genotoxicity) competes with a decrease (due to toxicity) resulting in a $S/N \approx 1$. The compound would therefore be interpreted as neither genotoxic or toxic (false negative result). The *prl* strain will clearly show a decrease in the light emission indicating that the compound is already toxic at the given dose. This is, at least for some doses, illustrated in figure 4 where nifuroxazide was taken as an example. It can be seen that the lower doses gave the greatest genotoxic response because of dose-dependent toxicity. The S/N curves for the *prl* strain clearly indicates that only the lowest doses up to 0.16 ppm were not toxic. Higher doses may show toxicity combined with genotoxicity (e.g., 0.32 ppm) or may be too toxic to show genotoxicity (highest doses).

Both the *recN2-4* and *prl* strains were shown

to be able to detect toxic concentrations of a compound. However, the *prl* strain is more valuable in toxicity testing as S/N curves can not be influenced by genotoxicity and hence reflect only toxicity (or its absence). The *prl* strain apparently provides a perfect tool for those who are only interested in toxicity assessment. We are at present comparing toxicity assessment of chemicals and complex mixtures with the *prl* strain and with the Microtox[®]-test. The latter is one of the most currently used and internationally accepted microbial toxicity tests that is also based on bioluminescence (Hasting, 1978; Férard et al., 1983). According to the limited data already available to us the VITOTOX[®]-test gives the same results as the Microtox[®]-test but the former is more easy to perform and is often much more sensitive. The VITOTOX[®]-test, or only its *prl*-element, may therefore also be considered as an extremely valuable toxicity test, at least when these preliminary results can be confirmed.

In conclusion it can be stressed that the TA104 *recN2-4* and TA104 *prl Salmonella typhimurium* strains provide very valuable genotoxicity and/or toxicity testsystems. Both strains should be used concomitantly for genotoxicity testing whereas the *prl* strain is only required for toxicity testing. It was shown that the VITOTOX[®]-test provides a very rapid (within 2-4 hours), and very sensitive answer with regard to the (geno)toxicity of chemicals and that it may for that reason be very useful in screening and in pre-screening of new chemicals and intermediate products. As testing is performed in 96-well plates it is at least possible to investigate 8 chemicals (with and without addition of a metabolic enzyme fraction S9) per day or 40 chemicals per week. Measurements occur automatically and data collection and data handling can

furthermore also almost completely be automated. Microplate robotisation should be able to multiply this rate with a factor of 10. Scale-up e.g. by increasing the amount of wells per plate is obvious. Labour costs are therefore
5 maximally reduced.

Finally, a supplementary and very important asset is that only very small volumes of the test compound are required (less than 20 mg). This is particularly important for the pharmaceutical industry where only a few
10 hundred milligrams of a compound are available in the *discovery phase*. It is just impossible to screen such new chemicals with (most) other test systems.

A deposit of the strain "pr1" has been made according to the Budapest Treaty under the deposit number
15 LMG P-18318 at the BCCM/LMG, Laboratorium voor Microbiologie - Bacteriënverzameling, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium.

REFERENCES

- Férard JF, et al., (1983): Application d'un test d'inhibition de luminescence bactérienne de l'étude toxicologique d'effluents complexes et de substances chimique. Revue Française des Sciences de l'Eau 2: 221-237.
- Hasting JW (1978): Bacterial bioluminescence: an overview. In: Methods in Enzymology. Academic Press, pp. 125-152.
- 10 Maron DM, Ames BN (1983): Revised methods for the Salmonella mutagenicity test. Mutation Res. 113: 173-215.
- Mergeay M, et al., (1985): *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriology, 162, 328-334.
- 15 Mersch-Sundermann V, et al., (1994): SOS induction in *Escherichia coli* and Salmonella mutagenicity: a comparison using 300 compounds. Mutagenesis 9: 205-224.
- 20 Mortelmans K et al., (1986): Salmonella mutagenicity tests: II. Results from the testing of 270 chemicals. Environ. Molec. Mutagen. 8, suppl. 7: 1-119.
- 25 Murray NE, et al., (1977): Lambdoid phages that simplify the recovery of in vitro recombinants. Mol.Gen.Genet. 150: 53-61.
- Quillardet P, Hofnung M (1993): The SOS chromotest: a review. Mutation Res. 297: 235-279.
- 30 Rostas K et al., (1987): Nucleotide sequence and LexA regulation of *Escherichia coli* recN gene. Nucl. Acids Res. 15: 5041-5049.

van der Lelie D et al., (1997): The VITO-TOX test, a SOS-bioluminescence *Salmonella typhimurium* test to measure genotoxicity kinetics. Mutation Res.389: 279-290.

5

van der Lelie D et al., (1997): Two-component regulatory system involved in transcriptional control of heavy-metal homeostasis in *Alcaligenes eutrophus*. Molecular Microbiology 23 (3): 439-503.

PCT

Original (for SUBMISSION) - printed on 12.04.1999 11:10:05 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.83 (updated 01.03.1999)
0-2	International Application No	PCT / B E 9 9 / 0 0 0 4 9
0-3	Applicant's or agent's file reference	P.VITO.12/WO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	21
1-2	line	13-17
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	Laboratorium voor Microbiologie - Bacteriënverzameling (BCCM/LMG)
1-3-2	Address of depositary institution	Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
1-3-3	Date of deposit	01 April 1998 (01.04.1998)
1-3-4	Accession Number	LMG P-18318
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications	NONE
	These indications will be submitted to the International Bureau later	

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	(12-04-1999) 12 AVR. 1999
0-4-1	Authorized officer	<i>Harmegnies</i>

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the International Bureau on:	
0-5-1	Authorized officer	

CLAIMS

1. A diagnostic system made of :

- a transformed micro-organism capable of an increased
5 reporter activity upon exposure to an environmental
insult, said micro-organism having a stress inducible
promoter sequence being operatively linked to a reporter
encoding nucleic acid sequence encoding a reporter
molecule resulting in a signal that can be assayed, and
10 of
- a transformed micro-organism having a constitutive and
non stress inducible promoter sequence being operatively
linked to a reporter encoding nucleic acid sequence
encoding a reporter molecule resulting in a signal that
15 can be assayed.

2. System according to claim 1, wherein the
signal is assayed as light production and/or colorimetric
modification.

3. System according to claim 1 or 2, wherein
20 the transformed bioluminescent micro-organism is *E. coli*.

4. System according to claim 1 or 2, wherein
the transformed bioluminescent micro-organism is a
Salmonella thyphimurium, preferably being a suitable Ames
test micro-organism, advantageously selected from the group
25 consisting of TA98, TA100, TA102, TA104, TA1535, TA1538,
TA7001 to TA7006, and TA7041 to TA7046, and/or having the
micro-organism deposit number LMG P-18318.

5. System according to any one of the
preceding claims 1 to 4, wherein the stress inducible
30 promoter sequence is selected from the group consisting of
groEL, dnaK, grpE, phoA, glnA, lon, lysU, rpoD, clpB, clpP,
uspA, katG, uvrA, frdA, micF, fabA, lac, his, sodA, sodB,
soi-28, recA, xthA, narG, recF, recJ, recN, recO, recQ,

ruv, uvrD, ars, cad, mer, pco and sfiA.

6. System according to any of the preceding claims 1 to 5, wherein the constitutive and non stress inducible promoter sequence is a sequence with a Sigma 70
5 consensus sequence (TTGACA(-35).....17/18 bpTATAAT(-10)) and whose transcription is not regulated positively or negatively at the promoter level.

7. System according to any one of the preceding claims 1 to 6, wherein the reporter encoding
10 nucleic acid sequence comprises the luciferase A and B genes or a luxAB translation fusion gene.

8. System according to any one of the preceding claims 1 to 7, wherein the reporter encoding nucleic acid sequence comprises the luciferase A and B
15 genes and the luciferase C, D and E genes required for producing the limiting fatty acid substrate that is used in recycling.

9. System according to any one of the preceding claims 1 to 8, wherein the environmental insult
20 is the presence of a genotoxic and/or a toxic compound into a sample.

10. Diagnostic kit comprising the elements of the diagnostic system according to any one of the preceding claims 1 to 9, and possibly the necessary additional
25 reactants, diluants and/or solid supports.

11. Method for the diagnostic of an environmental insult, preferably for determining the presence of a genotoxic and/or a toxic compound in a sample, comprising the steps of:

- 30 - exposing the diagnostic system according to any of the preceding claims 1 to 8 to said environmental insult, and
- measuring a signal of the transformed bioluminescent

micro-organism(s) of said diagnostic system.

12. Method for determining the kinetics of genotoxicity of a compound into a sample, wherein the measuring of luminescence of both inducible and
5 constitutive transformed micro-organisms occurs at multiple points in time, preferably continuously, and in addition carrying out the step of determining the signal-to-noise (S/N) ratio for the transformed micro-organisms at said point and time, dividing the S/N ratio of the inducible
10 micro-organism by that of the constitutive micro-organisms and plotting these data, said plotting representing the corrected kinetics of genotoxicity of the genotoxic compound in the sample.

13. Analysis method of an environmental
15 insult, comprising the steps of:

- performing the diagnostic of said environmental insult as described in claim 11,
- calculating the signal to noise ratio of both the transformed micro-organisms,
- 20 - classifying said environmental insult as toxic if at least one of the calculated signal to noise ratios is lower than 0.8,
- classifying said environmental insult as having no effect if the signal to noise ratio of the micro-
25 organism comprising the stress-inducible promoter sequence lies between 0.8 and 1.2, and
- classifying said environmental insult as inducing and genotoxic if the signal to noise ratio of the micro-
30 organism comprising the stress-inducible promoter sequence is higher than 1.2 and is at least 50 % higher than the signal to noise ratio of the micro-organism comprising the constitutive promoter sequence.

14. Installation for determining if a compound in a sample is genotoxic and/or toxic,

comprising a diagnostic system as in any of the claims 1
to 9 induced by said sample, a detection system
comprising a detection apparatus (2) able to assay the
signals from said diagnostic system, said detection
5 system being connected to a computer (1) programmed to
carry out the analysis method according to any of the
preceding the claims 11 to 13.

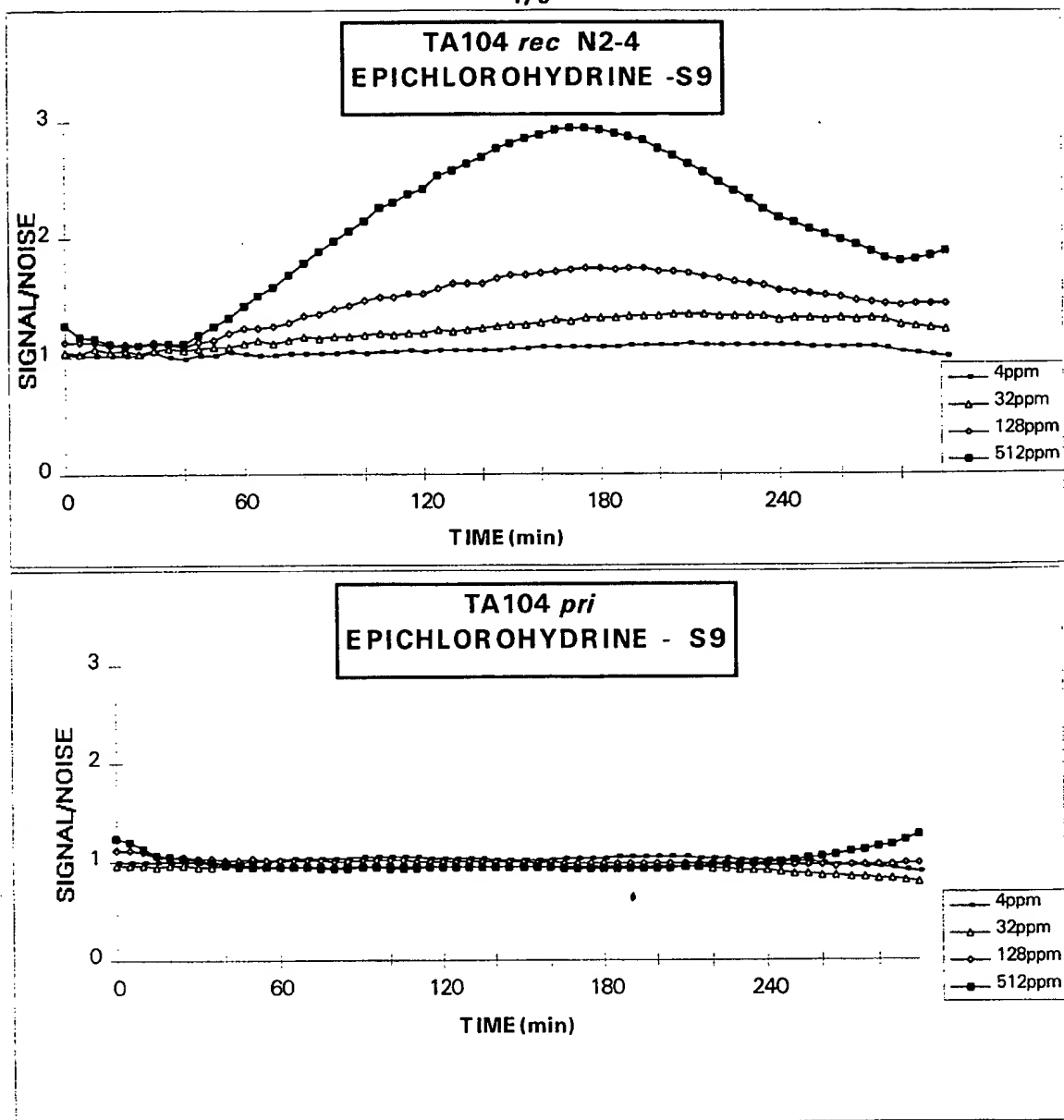


Fig. 1

2/6

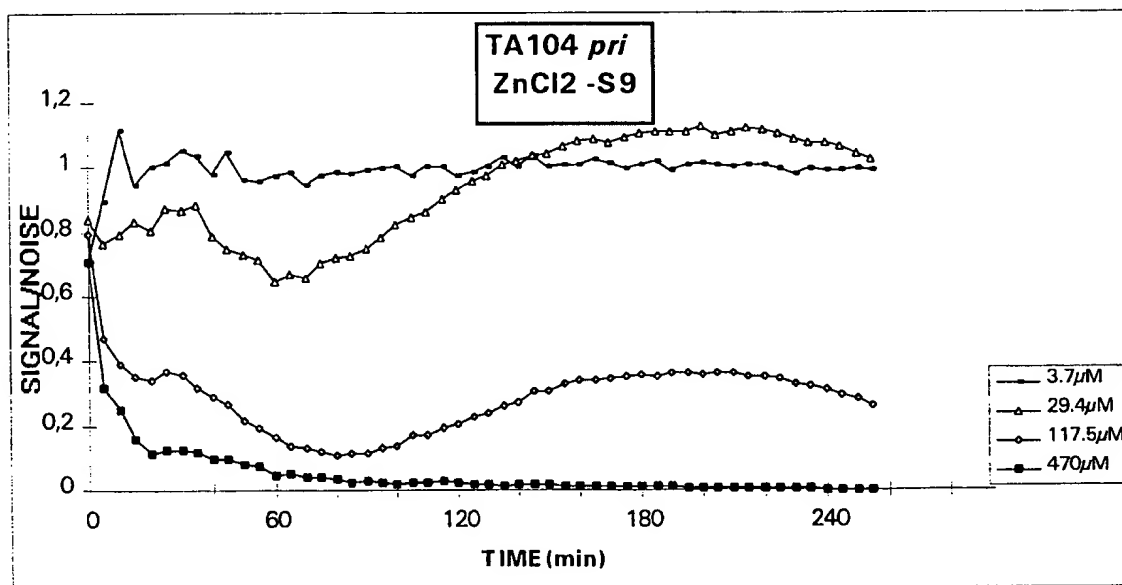
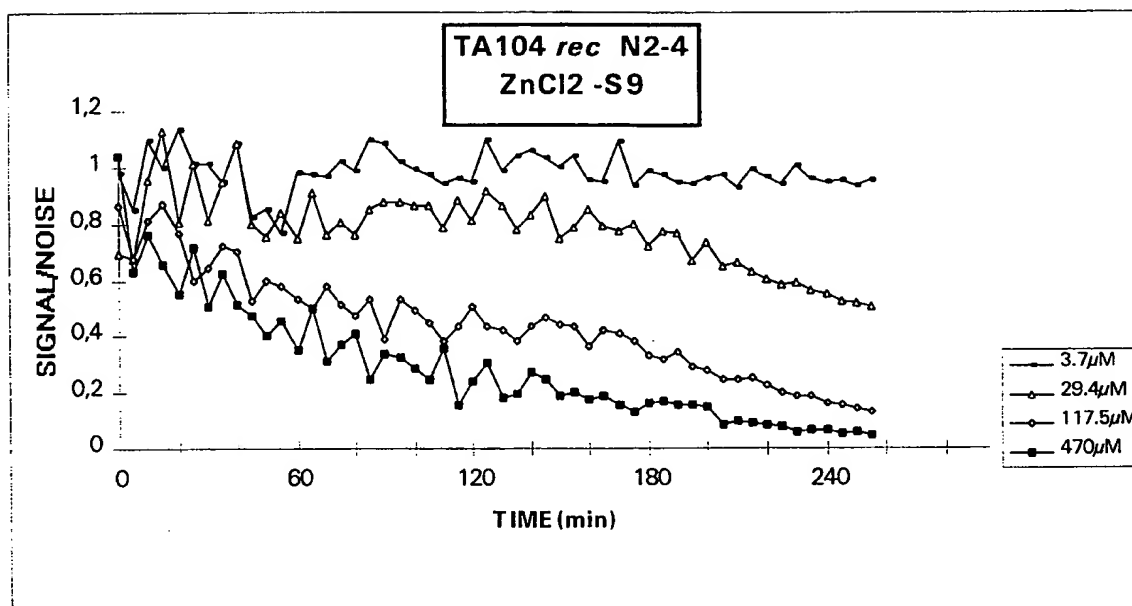


Fig.2

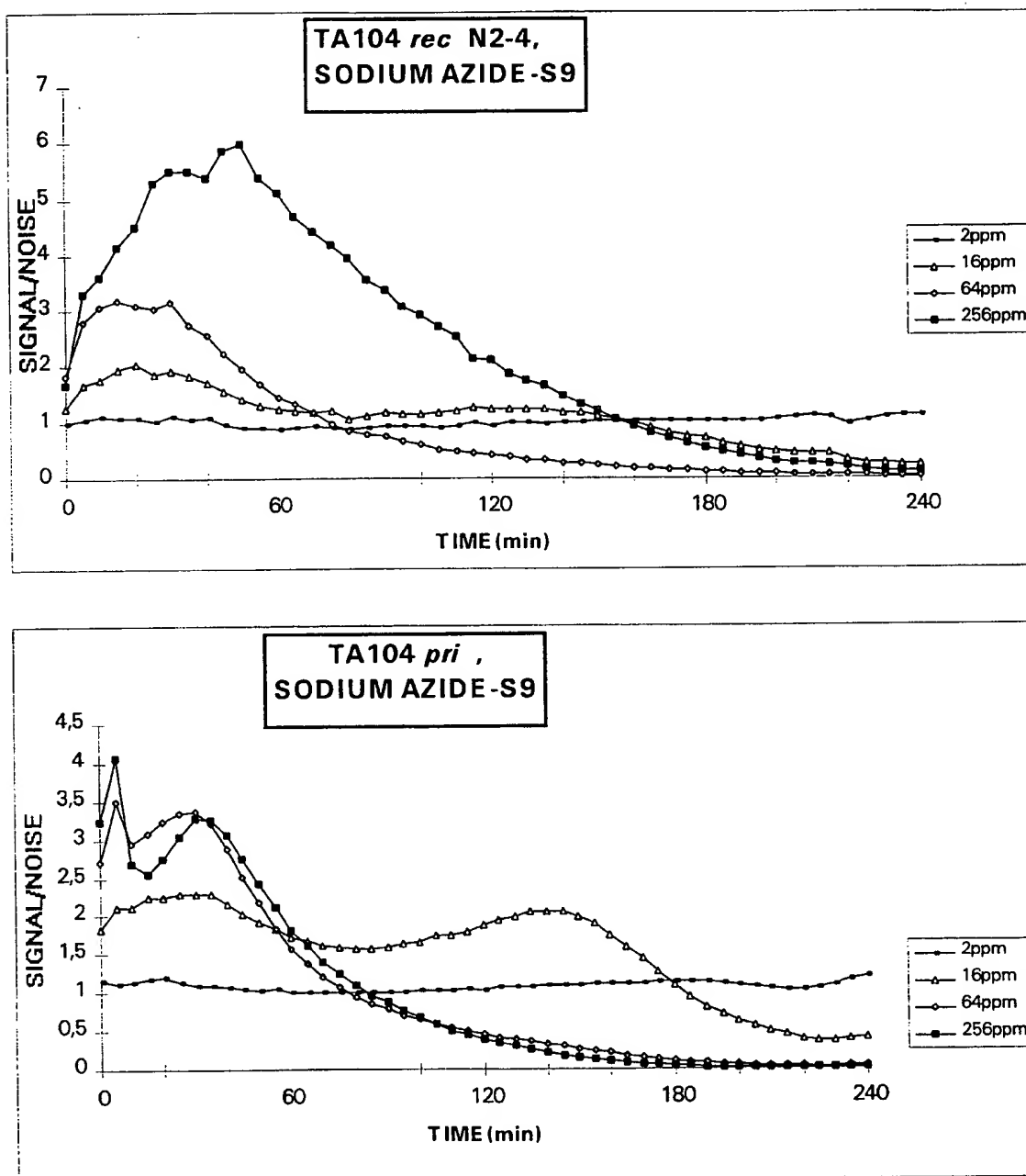


Fig. 3

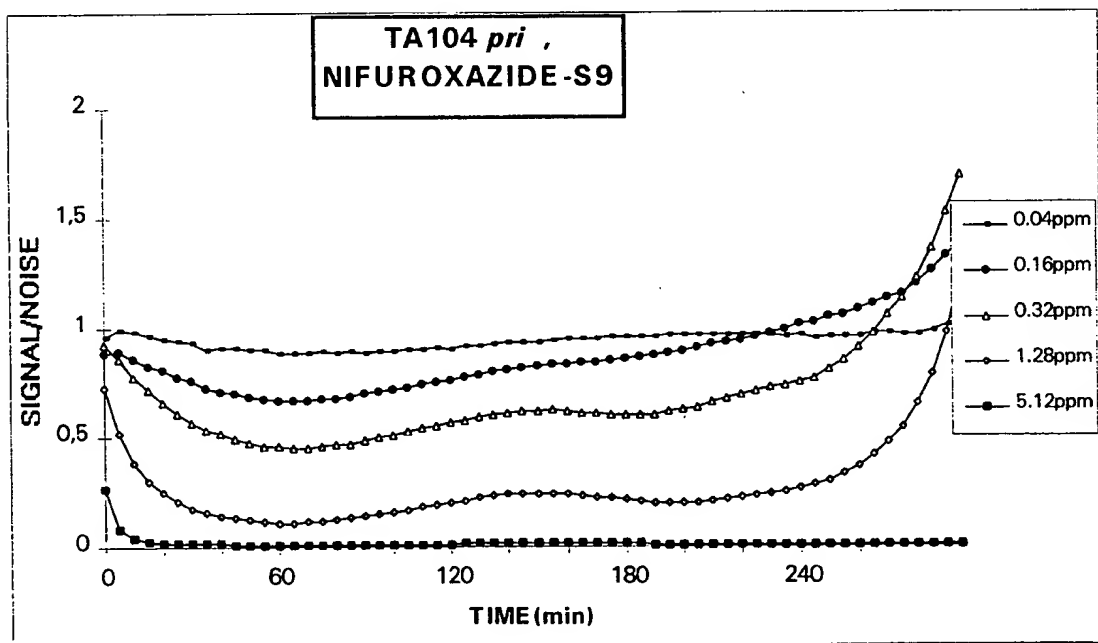
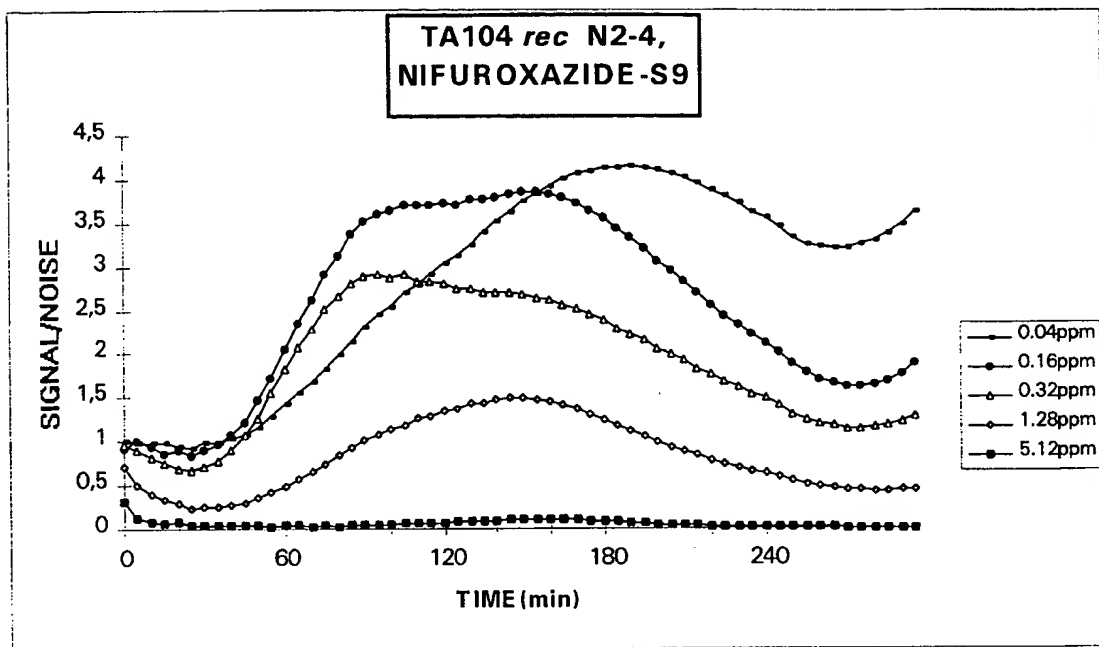


Fig. 4

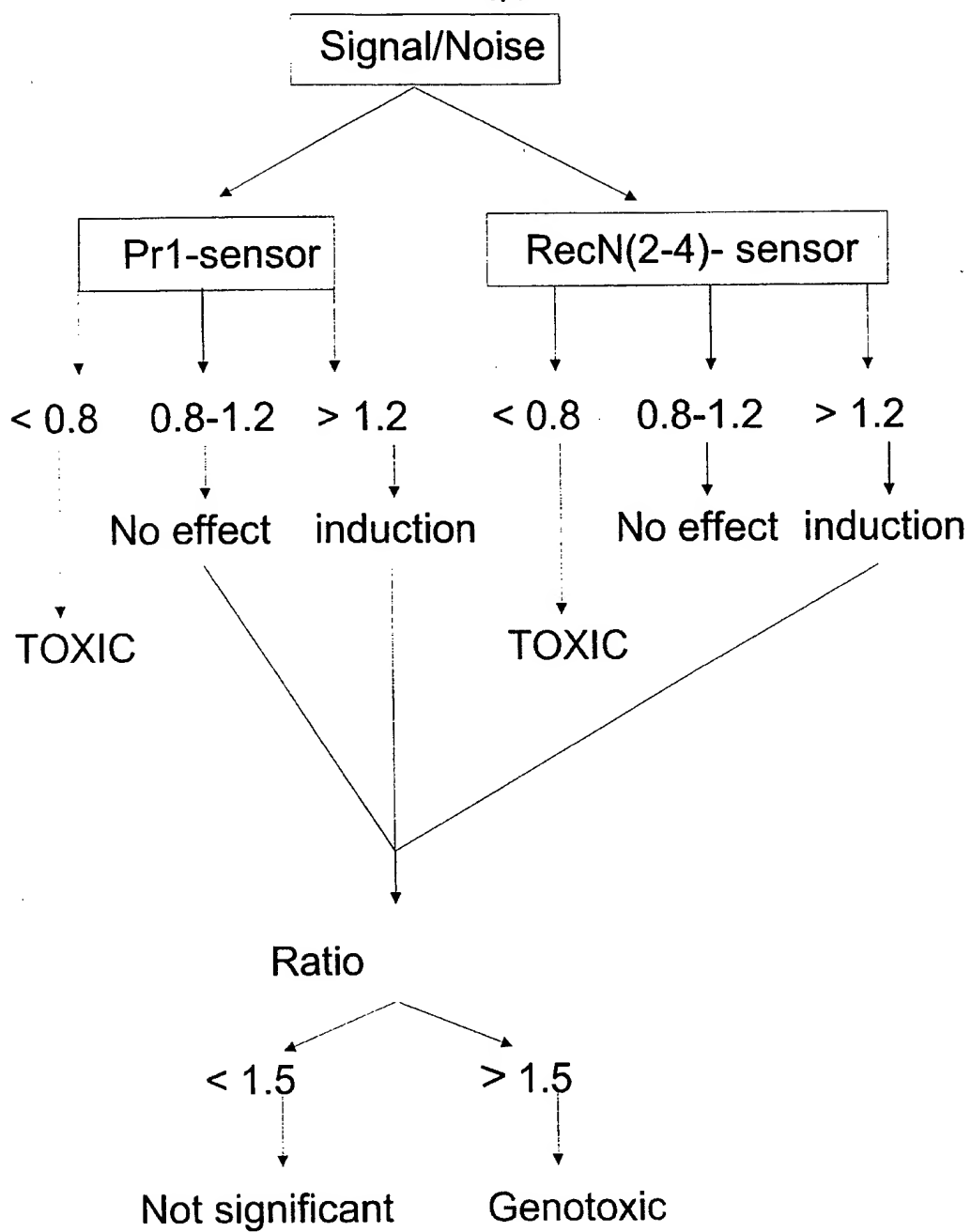


Fig. 5

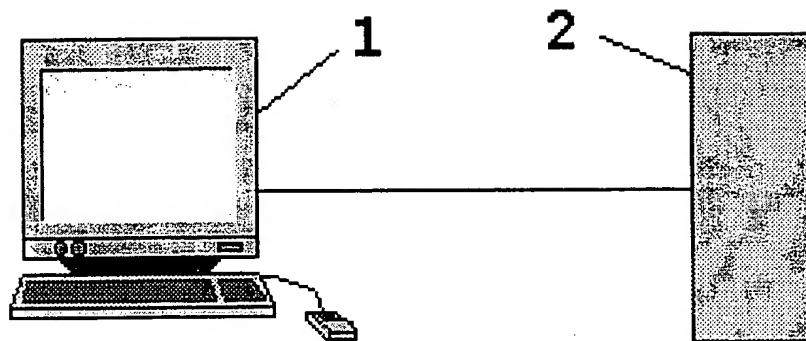


Fig. 6

**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure**

**Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG Identified at the bottom of next page**

International Form BCCM/LMG/BP/4/.....98-49...

To: Name of the depositor : Dr. Daniël van der Lelie

Address : Vlaamse Instelling voor Technologisch Onderzoek
Boeretang 200
B-2400 Mol

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

Salmonella typhimurium MA1007

I.2 Accession number given by the International Depositary Authority:

LMG P-18318

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

- ☐ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

April 1, 1998

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, Curator IDA

Date : April 9, 1998

**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure**

**Viability statement issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG identified on the following page**

International Form BCCM/LMG/BP/9/...98-49.....

To: Party to whom the viability statement is issued:

Name : Dr. Daniël van der Lelie

Address : Vlaamse Instelling voor Technologisch Onderzoek
Boeretang 200
B-2400 Mol

I. Depositor:

I.1 Name : Dr. Daniël van der Lelie

I.2 Address : Vlaamse Instelling voor Technologisch Onderzoek
Boeretang 200
B-2400 Mol

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-18318

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

April 1, 1998

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: April 2, 1998

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

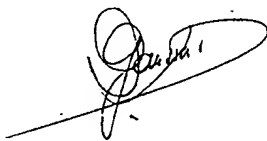
IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) :



Dr. D. Janssens, Curator IDA

Date : April 9, 1998

INTERNATIONAL SEARCH REPORT

International Application No

PCT/BE 99/00049

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 41251 A (VITO ;LELIE DANIEL V D (BE); BORREMANS BRIGITTE MARIE FRANC (BE);) 6 November 1997 see the whole document ---	1-14
Y	WO 94 13831 A (DYK TINA KANGAS VAN ;LAROSSA ROBERT ALAN (US); MAJARIAN WILLIAM RO) 23 June 1994 see the whole document --- -/--	1-14



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

29 June 1999

Date of mailing of the international search report

06/07/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Knehr, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/BE 99/00049

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>VAN DER LELIE D ET AL.: "The use of biosensors for environmental monitoring" RESEARCH IN MICROBIOLOGY, vol. 145, 1994, pages 67-74, XP002078145 see page 67, column 1, paragraph 1 - page 68, column 1, paragraph 1 *see especially page 72, paragraph 3 * see page 70, column 1, paragraph 5 - page 72, column 1, paragraph 3 ---</p>	1-5,7-14
Y	<p>WO 96 16187 A (DU PONT) 30 May 1996 see the whole document ---</p>	1-5,7-14
Y	<p>BELKIN S ET AL: "A panel of stress-responsive luminous bacteria for the detection of selected classes of toxicants" WATER RESEARCH, vol. 31, no. 12, December 1997, page 3009-3016 XP004098418 see the whole document ---</p>	1-3,5,7-14
Y	<p>RUPANI S ET AL.: "On-line monitoring of recombinant Escherichia coli in batch and continuous cultures using a grpE promotor bioluminescence reporter gene system" ABSTRACTS PAPERS OF THE AMERICAN CHEMICAL SOCIETY, vol. 207 Meet., no. Pt.1, 1994, page BIOT127 XP002078146 see abstract ---</p>	1-3,5,7-14
Y	<p>ORSER C S ET AL.: "Use of prokaryotic stress promoters as indicators of the mechanisms of chemical toxicity" IN VITRO TOXICOLOGY, vol. 8, no. 1, 1995, pages 71-85, XP002078147 see the whole document ---</p>	1-6,9,10,12
Y	<p>WO 94 01584 A (HARVARD COLLEGE ;FARR SPENCER B (US)) 20 January 1994 see abstract; claims 1-20; examples 6,9,12 ---</p>	1-6,9,10,12
A	<p>WO 92 15687 A (VITO) 17 September 1992 see the whole document ---</p>	
A	<p>EP 0 649 905 A (TOYODA CHUO KENKYUSHO KK) 26 April 1995 see the whole document -----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/BE 99/00049

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9741251	A	06-11-1997	AU 5648296 A EP 0907748 A	19-11-1997 14-04-1999
WO 9413831	A	23-06-1994	AT 152181 T AU 5730494 A CA 2150232 A DE 69310207 D DE 69310207 T EP 0673439 A ES 2102811 T JP 8504101 T US 5683868 A ZA 9309078 A	15-05-1997 04-07-1994 23-06-1994 28-05-1997 14-08-1997 27-09-1995 01-08-1997 07-05-1996 04-11-1997 05-06-1995
WO 9616187	A	30-05-1996	CA 2200702 A EP 0793729 A JP 10509049 T US 5731163 A	30-05-1996 10-09-1997 08-09-1998 24-03-1998
WO 9401584	A	20-01-1994	AT 162225 T AU 687353 B AU 4588493 A DE 69316368 D DE 69316368 T EP 0651825 A ES 2113546 T GR 3026639 T JP 8501930 T NO 950040 A US 5585232 A US 5589337 A	15-01-1998 26-02-1998 31-01-1994 19-02-1998 25-06-1998 10-05-1995 01-05-1998 31-07-1998 05-03-1996 06-03-1995 17-12-1996 31-12-1996
WO 9215687	A	17-09-1992	AT 165394 T DE 69225221 D DE 69225221 T EP 0573500 A ES 2117665 T US 5786162 A	15-05-1998 28-05-1998 17-09-1998 15-12-1993 16-08-1998 28-07-1998
EP 0649905	A	26-04-1995	JP 7227285 A JP 7163359 A DE 69413491 D DE 69413491 T US 5702883 A	29-08-1995 27-06-1995 29-10-1998 11-03-1995 30-12-1997